Flk-1 IS A RECEPTOR FOR VASCULAR ENDOTHELIAL GROWTH FACTOR

This application is a continuation-in-part of United States Application Serial No. 08/038,596, filed March 26, 1993, which is a continuation-in-part of United States Application Serial No. 07/975,750, filed November 13, 1992, both of which are incorporated by reference herein in their entirety.

1. INTRODUCTION

The present invention relates to the use of proteins, peptides and organic molecules capable of modulating FLK-1 receptor signal transduction in order to inhibit or promote angiogenesis and vasculogenesis. The invention is based, in part, on the demonstration that Flk-1 tyrosine kinase receptor expression is associated with endothelial cells and the identification of vascular endothelial growth factor (VEGF) as a high affinity ligand of Flk-1. These results indicate a major role for Flk-1 in the signaling system during vasculogenesis and angiogenesis. Engineering of host cells that express Flk-1 and the uses of expressed Flk-1 to evaluate and screen for drugs and analogs of VEGF involved in Flk-1 modulation by either agonist or antagonist activities is described.

The invention also relates to the use of FLK-1 ligands, including VEGF agonists and antagonists, in the treatment of disorders, including cancer, by modulating vasculogenesis and angiogenesis.

2. BACKGROUND OF THE INVENTION

Receptor tyrosine kinases comprise a large family of transmembrane receptors for polypeptide growth factors with diverse biological activities. Their

intrinsic tyrosine kinase function is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses

5 (Ullrich A. and Schlessinger, J., 1990, Cell 61:203-212).

A receptor tyrosine kinase cDNA, designated fetal liver kinase 1 (Flk-1), was cloned from mouse cell populations enriched for hematopoietic stem and

10 progenitor cells. The receptor was suggested to be involved in hematopoietic stem cell renewal (Matthews et al., 1991, Proc. Natl. Acad. Sci. USA 88:9026-9030). Sequence analysis of the Flk-1 clone revealed considerable homology with the c-Kit subfamily of

15 receptor kinases and in particular to the *Flt* gene product. These receptors all have in common an extracellular domain containing immunoglobulin-like structures.

The formation and spreading of blood vessels, or

20 vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus

25 luteum during ovulation and placental growth after pregnancy. Uncontrolled angiogenesis can be pathological such as in the growth of solid tumors that rely on vascularization for growth.

Angiogenesis involves the proliferation,

30 migration and infiltration of vascular endothelial cells, and is likely to be regulated by polypeptide growth factors. Several polypeptides with in vitro endothelial cell growth promoting activity have been identified. Examples include acidic and basic

35 fibroblastic growth factor, vascular endothelial

growth factor and placental growth factor. Although four distinct receptors for the different members of the FGF family have been characterized, none of these have as yet been reported to be expressed in blood

5 vessels in vivo.

While the FGFs appear to be mitogens for a large number of different cell types, VEGF has recently been reported to be an endothelial cell specific mitogen (Ferrara, N. and Henzel, W.J.,1989, Biochem. Biophys.

10 Res. Comm. 161:851-858). Recently, the fms-like tyrosine receptor, *flt*, was shown to have affinity for VEGF (DeVries,C. et al. ,1992, Science 255:989-991).

3. SUMMARY OF THE INVENTION

15 The present invention relates to the use of peptides, proteins and organic molecules capable of modulating FLK-1 receptor signal transduction in order to inhibit or promote angiogenesis and/or vasculogenesis. The present invention is based, in

20 part, on the discovery that the Flk-1 tyrosine kinase receptor is expressed on the surface of endothelial cells and the identification of vascular endothelial growth factor (VEGF) as a high affinity ligand of Flk-1. The role of endothelial cell proliferation and

25 migration during angiogenesis and vasculogenesis indicate an important role for Flk-1 in these processes. The invention is described by way of example for the murine Flk-1, however, the principles may be applied to other species including humans.

30 Pharmaceutical reagents designed to inhibit the Flk-1/VEGF interaction may be useful in inhibition of tumor growth. VEGF and/or VEGF agonists may be used to promote wound healing. The invention relates to expression systems designed to produce Flk-1 protein

35 and/or cell lines which express the Flk-1 receptor.

Expression of soluble recombinant Flk-1 protein may be used to screen peptide libraries for molecules that inhibit the Flk-1/VEGF interaction. Engineered cell lines expressing Flk-1 on their surface may be

5 advantageously used to screen and identify VEGF agonists and antagonists.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Comparison of the Flk-1 amino acid

10 sequence with related RTKs. Amino acid sequence comparison of Flk-1 with human KDR and rat TKr-C. A section of the sequence which is known for all three receptors is compared and only differences to the Flk-1 sequence are shown.

15 FIGS. 2A and 2B. Northern blot analysis of Flk-1 gene expression. FIG 2A. Expression of Flk-1 RNA in day 9.5 to day 18.5 mouse embryos. Samples (10 μg) of total RNA from whole mouse embryos were analyzed in each lane. Positions of 28S and 18S ribosomal RNAs

20 are marked.

FIG. 2B Expression of Flk-1 mRNA in postnatal day 4 and adult brain in comparison with capillary fragments from postnatal day 4 brain. 1μg of poly (A$^+$) RNA was loaded on each lane. The 5' 2619 bp of the Flk-1 cDNA

25 were used as a probe. Control hybridization with a GAPDH cDNA probe is shown in the lower panel.

FIGS. 3A, 3B and 3C. Abundant Flk-1 gene expression in embryonic tissues. *In situ* hybridization analysis of Flk-1 expression in day 14.5

30 mouse embryo. FIG. 3A Bright field illumination of a parasagittal section through the whole embryo hybridized with a ^{35}S-labeled antisense probe (5' 2619 bp). FIG. 3B Dark field illumination of the same section. FIG. 3C Control hybridization of an adjacent

35 section with a sense probe. Abbreviations: Ao, aorta;

At, atrium; L, lung; Li, liver; Ma, mandible; Mn,
meninges; Ms. mesencephalon; T, telencephalon; V,
ventricle; Vt, vertebrae.

FIGS. 4A, 4B, 4C, 4D and 4E. Expression of Flk-1
5 RNA in embryonic organs is restricted to specific
cells. Expression of Flk-1 RNA in a day 14.5 mouse
embryo at higher magnification. FIG. 4A The heart
region was probed with a ^{35}S-labeled antisense probe.
FIG. 4B Adjacent section hybridized with the sense
10 probe.
FIG. 4C Part of the aorta wall shown on the cellular
level. The endothelial cell-layer is indicated by an
arrow. FIG. 4D The lung, probed with the Flk-1
antisense probe. FIG. 4E Control hybridization of an
15 adjacent section hybridized with the sense probe.
Abbreviations: At, atrium; B, bronchus; Ed,
endothelial cell layer; En, endocardium; L, lung, Li,
liver; Lu, lumina of the aorta; Ml, muscular; My,
myocardium.
20 FIGS. 5A, 5B, 5C and 5D. Flk-1 gene expression
in the brain of the developing mouse. *In situ*
hybridization analysis of Flk-1 gene expression in the
brain at different developmental stages. All sections
were probed with the Flk-1 antisense probe. FIG. 5A
25 Sagittal section of the telencephalon of a day 11.5
mouse embryo. A single blood vessel expressing Flk-
1, which sprouts from the meninges into the
neuroectoderm, is indicated by an arrow. FIG. 5B
Sagittal sections of the brain of embryo day 14.5 and
30 FIG. 5C of postnatal day 4. Shown are regions of the
mesencephalon. Branching capillaries and blood
vessels expressing Flk-1 are indicated by an arrow.
FIG. D Sagittal section of an adult brain; a region of
the mesencephalon is shown. Cells expressing Flk-1
35

are indicated by an arrow. Abbreviations: M,
meninges; V, ventricle;

FIG. 6A and 6B. Expression of Flk-1 in the
choroid plexus of adult brain. FIG. 6A Darkfield
5   illumination of the choroid plexus of an adult mouse
brain hybridized with Flk-1 antisense probe. FIG. 6B
Choroid plexus shown at a higher magnification.
Arrows indicate single cells, which show strong
expression of Flk-1. Abbreviations: CP, choroid
10 plexus; E, ependyme; Ep, epithelial cells; V,
ventricle.

FIGS. 7A, 7B, 7C and 7D. Flk-1 is expressed in
the glomeruli of the kidney. FIG. 7A Parasagittal
section of a 4-day postnatal kidney, hybridized with
15 the Flk-1 antisense probe. Hybridization signal
accumulates in the glomeruli, as indicated by
arrowheads. FIG. 7B Control hybridization of an
adjacent section with the sense probe. FIG. 7C
Sagittal section of an adult kidney probed with Flk-1.
20 Arrowheads indicate glomeruli. FIG. 7D Glomerulus of
an adult kidney at a higher magnification. The arrows
in (A) and (D) indicate cells aligned in strands in
the juxtaglomerular region expressing Flk-1.

FIGS. 8A, 8b, 8C and 8D. *In situ* hybridization
25 analysis of Flk-1 expression in early embryos and
extraembryonic tissues. FIG. 8A Sagittal section of a
day 8.5 mouse embryo in the maternal deciduum probed
with Flk-1. FIG. 8B Higher magnification of the
deciduum. Arrowheads indicate the endothelium of
30 maternal blood vessels strongly expressing Flk-1 RNA.
FIG. 8C High magnification of the yolk sac and the
trophectoderm of a day 9.5 mouse embryo. FIG. 8D High
magnification of a blood island. Abbreviations:
A, allantois; Bi, blood island; Bv, maternal blood
35 vessel; D, deciduum; En, endodermal layer of yolk sac;

M, mesenchyme; Ms, mesodermal layer of yolk sac; NF, neural fold; T, trophoblast; Y, yolk sac.

FIGS. 9A and 9B. Flk-1 is a receptor for VEGF. FIG. 9A Cross linking of ^{125}I-VEGF to COS cells transiently expressing the Flk-1 receptor and control cells were incubated with ^{125}I-VEGF at 4°C overnight, then washed twice with phosphate buffered saline (PBS) and exposed to 0.5 mM of the cross linking agent DSS in PBS for 1 hour at 4°C. The cells were lysed, Flk-1 receptor immunoprecipitated, and analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Molecular size markers are indicated in kilodaltons. FIG. 9B Specific binding of ^{125}I-VEGF to COS cells expressing Flk-1. COS cells transiently expressing Flk-1 were removed from the plate and resuspended in binding medium (DMEM, 25 mM Hepes, 0.15% gelatin). Binding was performed at 15°C for 90 minutes in a total volume of 0.5 ml containing 2x10^5 cells, 15,000 cpm ^{125}I-VEGF, and the indicated concentrations of unlabeled ligand. The cells were washed twice with PBS / 0.1% BSA and counted in a gamma counter.

FIG. 10. VEGF-induced autophosphorylation of Flk-1. COS cells transiently expressing Flk-1 receptor and control cells were starved for 24 hours in DMEM containing 0.5% fetal calf serum and then stimulated with VEGF for 10 minutes as indicated. The cells were solubilized, Flk-1 receptor immunoprecipitated with a polyclonal antibody against its C-terminus, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blot was probed with antiphosphotyrosine antibodies (5B2). The protein bands were visualized by using a horseradish-peroxidase coupled secondary antibody and BCL™ (Amersham) detection assay.

FIGS. 11A and 11B. Nucleotide Sequence of Murine Flk-1.

FIGS. 12A and 12B. Plasmid Maps of retroviral vector constructs. FIG. 12A. pLXSN Flk-1 TM cl.1 and pLXSN Flk-1 TM cl.3, clonal isolates of pLXSN Flk-1 TM, contain Flk-1 amino acids 1 through 806 and lack 561 C-terminal amino acids of the intracellular kinase domain. FIG. 12B. pNTK-cfms-TM contains the 541 N-terminal amino acids of the CSF-1 receptor/c-fms.

FIG. 13. Inhibition of C6 glioblastoma tumor growth by transdominant-negative inhibition of Flk-1. C6 cells were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cells lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-negative c-fms mutant (c-fms TM) as a control. Beginning at the time when the first tumors appeared, tumor volumes were measured every 2 to 3 days to obtain a growth curve. Each group is represented by four mice.

FIG. 14. A second experiment showing inhibition of C6 glioblastoma tumor growth by transdominant-negative inhibition of Flk-1. C6 cells were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cell lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-negative c-fms mutant (cfms TM) as a control. Beginning at the time when the first tumor appeared, tumor volumes were measured every 2 to 3 days to obtain growth curve. Each group is represented by four mice.

FIG. 15. Inhibition of C6 glioblastoma tumor growth by localized injection of retroviral

supernatants. 1×10^6 cells were subcutaneously implanted in nude mice. Starting at day 5 after implantation (denoted by the arrow), the growing tumors were treated by injection of 100 μl retroviral

5 supernatants (about 105 virus particles) into the site of tumor implantation. Tumor volumes were measured twice a week.

FIG. 16A and 16B. Inhibition of C6 glioblastoma tumor growth by localized injection of retroviral

10 supernatants. C6 cells implanted intercranially in rats, either alone or co-implanted with virus producing cells expressing the Flk-1 TM (transdominant negative) mutant. Each group is represented by 8 rats. Cell numbers are as indicated in Example

15 6.1.13.

FIG. 16A shows the distribution of tumor size in each rat. FIG. 16B shows the median tumor area for each of the two groups of rats.

FIG. 17. Inhibition of VEGF stimulatory activity

20 of Flk-1. Test compounds and VEGF were co-incubated on cells expressing the Flk-1 receptor. The level of tyrosine phosphorylation was measured in a Western blot format using an antiphosphotyrosine antibody. Compound A14 completely inhibited the ability of VEGF

25 to stimulate autophosphorylation of Flk-1. (Compounds A7, A8, and A10 were toxic to these cells resulting in cell death.)

5. DETAILED DESCRIPTION OF THE INVENTION

30 The present invention relates to the use of peptides, proteins, and organic molecules capable of modulating FLK-1 receptor signal transduction in order to inhibit or promote angiogenesis and/or vasculogenesis. More specifically, the invention is

35 directed to VEGF, a natural ligand for Flk-1, as well

as VEGF agonists and antagonists, anti-VEGF and anti-Flk-1 antibodies, and VEGF and Flk-1 encoding polynucleotides, all of which may find use in modulating Flk-1 signal transduction. Another aspect

5 of the invention relates to the use of Flk-1 expressing cells to evaluate and screen proteins, peptides, and organic compounds that may be involved in Flk-1 receptor activation, regulation and/or uncoupling. Such regulators of Flk-1 may be used

10 therapeutically. For example, agonists of VEGF may be used in processes such as wound healing; in contrast, antagonists of VEGF may be used in the treatment of tumors that rely on vascularization for growth.

The invention, is based, in part, on results from

15 in situ-hybridization and Northern blot analyses indicating that Flk-1 is an endothelial cell specific RTK. In addition, cross-linking experiments have shown Flk-1 to be a high affinity receptor for vascular endothelial growth factor (VEGF), indicating

20 that Flk-1 plays a crucial role in the development and differentiation of hemangioblast and in subsequent endothelial cell growth during vasculogenesis and angiogenesis.

The invention is based, also, on the discovery

25 that expression of a transdominant-negative mutant form of the Flk-1 molecule can inhibit the biological activity of the endogenous wild type Flk-1. Experiments are described herein, in which tumor cells and cells expressing retrovirally-encoded truncated,

30 signalling-incompetent Flk-1 receptor were injected into mice. In these experiments, the cells producing a recombinant retrovirus encoding a truncated Flk-1 receptor were either co-injected with the tumor cells or injected 5 days after injection of the tumor cells.

35 Inhibition of vasculogenesis and growth of the

injected tumor cells was observed in mice expressing the truncated form of the Flk-1 receptor. Inhibition of tumor growth observed when the truncated Flk-1 receptor was injected 5 days after injection of the

5 tumor cells indicates that even established tumors may be suppressed by Flk-1 dominant-negative action. Thus, the invention provides a method of inhibiting the biological activity of signalling-competent Flk-1 receptors comprising introducing a signalling-

10 incompetent Flk-1 receptor mutant into or in the vicinity of cells expressing such signalling-competent Flk-1 receptors. Accordingly, expression of transdominant negative forms of the Flk-1 molecule may be useful for treatment of diseases resulting from

15 VEGF and/or Flk-1 mediated, abnormal proliferation of blood vessels, such as rheumatoid arthritis, retinopathies and growth of solid tumors.

As explained in the working examples, infra, the polymerase chain reaction (PCR) method was used to

20 isolate new receptor tyrosine kinases specifically expressed in post-implantation embryos and endothelial cells. One such clone was found to encode a RTK that had almost identical sequence homology with the previously identified cDNA clone isolated from

25 populations of cells enriched for hematopoietic cells and designated fetal liver kinase-1 (Flk-1) (Matthews et al., 1991, Proc. Natl. Acad Sci. U.S.A. 88:9026-9030) (FIGS. 11A and 11B).

For clarity of discussion, the invention is

30 described in the subsections below by way of example for the murine Flk-1. However, the principles may be analogously applied to clone and express the Flk-1 of other species including humans.

35

5.1. THE Flk-1 CODING SEQUENCE

The nucleotide coding sequence and deduced amino acid sequence of the murine Flk-1 gene is depicted in Figures 11A and 11B (SEQ. ID NO. 1) and has recently been described in Matthews et al., 1991, Proc. Natl. Acad. Sci. U.S.A., 88:9026-9030. In accordance with the invention, the nucleotide sequence of the Flk-1 protein or its functional equivalent in mammals, including humans, can be used to generate recombinant molecules which direct the expression of Flk-1; hereinafter, this receptor will be referred to as "Flk-1", regardless of the species from which it is derived.

In a specific embodiment described herein, the murine Flk-1 gene was isolated by performing a polymerase chain reaction (PCR) using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of receptor tyrosine kinases (Hanks et al., 1988,) As a template, DNA from a λgt10 cDNA library prepared from day 8.5 mouse embryos, was used. In a parallel approach, similar primers were used to amplify RTK cDNA sequences from capillary endothelial cells that had been isolated from the brains of post-natal day 4-8 mice. This is a time when brain endothelial cell proliferation is maximal. Both approaches yielded cDNA sequences encoding the recently described fetal liver RTK, Flk-1 (Matthews et al., 1991). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich and Schlessinger) which contain immunoglobulin-like repeats in their extracellular domains (FIG. 1).

The invention also relates to Flk-1 genes isolated from other species, including humans, in

which Flk-1 activity exists. Members of the Flk-1
family are defined herein as those receptors that bind
VEGF or fragments of the peptide. Such receptors may
demonstrate about 80% homology at the amino acid level
5   in substantial stretches of DNA sequence. A
bacteriophage cDNA library may be screened, under
conditions of reduced stringency, using a
radioactively labeled fragment of the mouse Flk-1
clone. Alternatively the mouse Flk-1 sequence can be
10   used to design degenerate or fully degenerate
oligonucleotide probes which can be used as PCR probes
or to screen bacteriophage cDNA libraries. A
polymerase chain reaction (PCR) based strategy may be
used to clone human Flk-1. Two pools of degenerate
15   oligonucleotides, corresponding to a conserved motif
between the mouse Flk-1 and receptor tyrosine kinases,
may be designed to serve as primers in a PCR reaction.
The template for the reaction is cDNA obtained by
reverse transcription of mRNA prepared from cell lines
20   or tissue known to express human Flk-1. The PCR
product may be subcloned and sequenced to insure that
the amplified sequences represent the Flk-1 sequences.
The PCR fragment may be used to isolate a full length
Flk-1 cDNA clone by radioactively labeling the
25   amplified fragment and screening a bacteriophage cDNA
library. Alternatively, the labeled fragment may be
used to screen a genomic library. For a review of
cloning strategies which may be used, see e.g.,
Maniatis, 1989, Molecular Cloning, A Laboratory
30   Manual, Cold Springs Harbor Press, N.Y.; and Ausubel
et al., 1989, Current Protocols in Molecular Biology,
(Green Publishing Associates and Wiley Interscience,
N.Y.)

      Isolation of a human Flk-1 cDNA may also be
35   achieved by construction of a cDNA library in a

mammalian expression vector such as pcDNA1, that contains SV40 origin of replication sequences which permit high copy number expression of plasmids when transferred into COS cells. The expression of Flk-1

5 on the surface of transfected COS cells may be detected in a number of ways, including the use of a labeled ligand such as VEGF or a VEGF agonist labeled with a radiolabel, fluorescent label or an enzyme. Cells expressing the human Flk-1 may be enriched by

10 subjecting transfected cells to a FACS (fluorescent activated cell sorter) sort.

In accordance with the invention, Flk-1 nucleotide sequences which encode Flk-1, peptide fragments of Flk-1, Flk-1 fusion proteins or

15 functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of Flk-1 protein or a functional equivalent thereof, in appropriate host cells. Alternatively, nucleotide sequences which hybridize to portions of the Flk-1

20 sequence may also be used in nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially

25 the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the Flk-1 protein. Such DNA sequences include those which are capable of hybridizing to the murine Flk-1 sequence under

30 stringent conditions.

Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a

35 functionally equivalent gene product. The gene

product itself may contain deletions, additions or
substitutions of amino acid residues within the Flk-1
sequence, which result in a silent change thus produ-
cing a functionally equivalent Flk-1. Such amino acid
5 substitutions may be made on the basis of similarity
in polarity, charge, solubility, hydrophobicity,
hydrophilicity, and/or the amphipatic nature of the
residues involved. For example, negatively charged
amino acids include aspartic acid and glutamic acid;
10 positively charged amino acids include lysine and
arginine; amino acids with uncharged polar head groups
having similar hydrophilicity values include the
following: leucine, isoleucine, valine; glycine,
analine; asparagine, glutamine; serine, threonine;
15 phenylalanine, tyrosine. As used herein, a function-
ally equivalent Flk-1 refers to a receptor which binds
to VEGF or fragments, but not necessarily with the
same binding affinity of its counterpart native Flk-1.

The DNA sequences of the invention may be engi-
20 neered in order to alter the Flk-1 coding sequence for
a variety of ends including but not limited to altera-
tions which modify processing and expression of the
gene product. For example, mutations may be intro-
duced using techniques which are well known in the
25 art, e.g. site-directed mutagenesis, to insert new
restriction sites, to alter glycosylation patterns,
phosphorylation, etc. For example, in certain
expression systems such as yeast, host cells may over
glycosylate the gene product. When using such expres-
30 sion systems it may be preferable to alter the Flk-1
coding sequence to eliminate any N-linked glycosyla-
tion site.

In another embodiment of the invention, the Flk-1
or a modified Flk-1 sequence may be ligated to a
35 heterologous sequence to encode a fusion protein. For

example, for screening of peptide libraries it may be useful to encode a chimeric Flk-1 protein expressing a heterologous epitope that is recognized by a commercially available antibody. A fusion protein may
5 also be engineered to contain a cleavage site located between the Flk-1 sequence and the heterologous protein sequence, so that the Flk-1 can be cleaved away from the heterologous moiety.

In an alternate embodiment of the invention, the
10 coding sequence of Flk-1 could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, Nuc. Acids Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers,
15 1980, Tetrahedron Letters 21:719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize the Flk-1 amino acid sequence in whole or in part. For example, peptides can be
20 synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high perform- ance liquid chromatography. (E.g., see Creighton, 1983, Proteins Structures And Molecular Principles, W.H. Freeman and Co., N.Y. pp. 50-60). The composi-
25 tion of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49).
30

5.2. EXPRESSION OF Flk-1 RECEPTOR AND GENERATION
OF CELL LINES THAT EXPRESS Flk-1

In order to express a biologically active Flk-1, the nucleotide sequence coding for Flk-1, or a func-
35 tional equivalent as described in Section 5.1 supra,

is inserted into an appropriate expression vector,
i.e., a vector which contains the necessary elements
for the transcription and translation of the inserted
coding sequence. The Flk-1 gene products as well as
5   host cells or cell lines transfected or transformed
with recombinant Flk-1 expression vectors can be used
for a variety of purposes. These include but are not
limited to generating antibodies (i.e., monoclonal or
polyclonal) that bind to the receptor, including those
10   that competitively inhibit binding of VEGF and
"neutralize" activity of Flk-1 and the screening and
selection of VEGF analogs or drugs that act via the
Flk-1 receptor; etc.

15   5.2.1.   EXPRESSION SYSTEMS
        Methods which are well known to those skilled in
the art can be used to construct expression vectors
containing the Flk-1 coding sequence and appropriate
transcriptional/translational control signals. These
20   methods include in vitro recombinant DNA techniques,
synthetic techniques and in vivo recombination/genetic
recombination. See, for example, the techniques
described in Maniatis et al., 1989, Molecular Cloning
A Laboratory Manual, Cold Spring Harbor Laboratory,
25   N.Y. and Ausubel et al., 1989, Current Protocols in
Molecular Biology, Greene Publishing Associates and
Wiley Interscience, N.Y.
        A variety of host-expression vector systems may
be utilized to express the Flk-1 coding sequence.
30   These include but are not limited to microorganisms
such as bacteria transformed with recombinant
bacteriophage DNA, plasmid DNA or cosmid DNA expres-
sion vectors containing the Flk-1 coding sequence;
yeast transformed with recombinant yeast expression
35   vectors containing the Flk-1 coding sequence; insect

cell systems infected with recombinant virus expres-
sion vectors (e.g., baculovirus) containing the Flk-1
coding sequence; plant cell systems infected with
recombinant virus expression vectors (e.g.,
5 cauliflower mosaic virus, CaMV; tobacco mosaic virus,
TMV) or transformed with recombinant plasmid expres-
sion vectors (e.g., Ti plasmid) containing the Flk-1
coding sequence; or animal cell systems infected with
recombinant virus expression vectors (e.g.,
10 adenovirus, vaccinia virus) including cell lines
engineered to contain multiple copies of the Flk-1 DNA
either stably amplified (CHO/dhfr) or unstably
amplified in double-minute chromosomes (e.g., murine
cell lines).

15   The expression elements of these systems vary in
their strength and specificities. Depending on the
host/vector system utilized, any of a number of
suitable transcription and translation elements,
including constitutive and inducible promoters, may be
20 used in the expression vector. For example, when
cloning in bacterial systems, inducible promoters such
as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac
hybrid promoter) and the like may be used; when
cloning in insect cell systems, promoters such as the
25 baculovirus polyhedrin promoter may be used; when
cloning in plant cell systems, promoters derived from
the genome of plant cells (e.g., heat shock promoters;
the promoter for the small subunit of RUBISCO; the
promoter for the chlorophyll a/b binding protein) or
30 from plant viruses (e.g., the 35S RNA promoter of
CaMV; the coat protein promoter of TMV) may be used;
when cloning in mammalian cell systems, promoters
derived from the genome of mammalian cells (e.g.,
metallothionein promoter) or from mammalian viruses
35 (e.g., the adenovirus late promoter; the vaccinia

virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the Flk-1 DNA SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

5   In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the Flk-1 expressed. For example, when large quantities of Flk-1 are to be produced for the generation of antibodies or to screen

10 peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791),

15 in which the Flk-1 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol.

20 Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to

25 glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

30   In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al.,

35 1987, Expression and Secretion Vectors for Yeast, in

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Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA
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5 Methods in Enzymology, Eds. Berger & Kimmel, Acad.
Press, N.Y., Vol. 152, pp. 673-684; and The Molecular
Biology of the Yeast Saccharomyces, 1982, Eds.
Strathern et al., Cold Spring Harbor Press, Vols. I
and II.

10   In cases where plant expression vectors are used,
the expression of the Flk-1 coding sequence may be
driven by any of a number of promoters. For example,
viral promoters such as the 35S RNA and 19S RNA
promoters of CaMV (Brisson et al., 1984, Nature

15 310:511-514), or the coat protein promoter of TMV
(Takamatsu et al., 1987, EMBO J. 6:307-311) may be
used; alternatively, plant promoters such as the small
subunit of RUBISCO (Coruzzi et al., 1984, EMBO J.
3:1671-1680; Broglie et al., 1984, Science 224:838-

20 843); or heat shock promoters, e.g., soybean hsp17.5-E
or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol.
6:559-565) may be used. These constructs can be
introduced into plant cells using Ti plasmids, Ri
plasmids, plant virus vectors, direct DNA transfor-

25 mation, microinjection, electroporation, etc. For
reviews of such techniques see, for example, Weissbach
& Weissbach, 1988, Methods for Plant Molecular
Biology, Academic Press, NY, Section VIII, pp. 421-
463; and Grierson & Corey, 1988, Plant Molecular

30 Biology, 2d Ed., Blackie, London, Ch. 7-9.

       An alternative expression system which could be
used to express Flk-1 is an insect system. In one
such system, Autographa californica nuclear
polyhidrosis virus (AcNPV) is used as a vector to

35 express foreign genes. The virus grows in Spodoptera

frugiperda cells. The Flk-1 coding sequence may be
cloned into non-essential regions (for example the
polyhedrin gene) of the virus and placed under control
of an AcNPV promoter (for example the polyhedrin
5   promoter). Successful insertion of the Flk-1 coding
sequence will result in inactivation of the polyhedrin
gene and production of non-occluded recombinant virus
(i.e., virus lacking the proteinaceous coat coded for
by the polyhedrin gene). These recombinant viruses
10   are then used to infect Spodoptera frugiperda cells in
which the inserted gene is expressed. (E.g., see
Smith et al., 1983, J. Viol. 46:584; Smith, U.S.
Patent No. 4,215,051).

      In mammalian host cells, a number of viral based
15   expression systems may be utilized. In cases where an
adenovirus is used as an expression vector, the Flk-1
coding sequence may be ligated to an adenovirus
transcription/translation control complex, e.g., the
late promoter and tripartite leader sequence. This
20   chimeric gene may then be inserted in the adenovirus
genome by in vitro or in vivo recombination. Inser-
tion in a non-essential region of the viral genome
(e.g., region E1 or E3) will result in a recombinant
virus that is viable and capable of expressing Flk-1
25   in infected hosts. (E.g., See Logan & Shenk, 1984,
Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alterna-
tively, the vaccinia 7.5K promoter may be used. (See,
e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci.
(USA) 79:7415-7419; Mackett et al., 1984, J. Virol.
30   49:857-864; Panicali et al., 1982, Proc. Natl. Acad.
Sci. 79:4927-4931).

      Specific initiation signals may also be required
for efficient translation of inserted Flk-1 coding
sequences. These signals include the ATG initiation
35   codon and adjacent sequences. In cases where the

entire Flk-1 gene, including its own initiation codon
and adjacent sequences, is inserted into the appro-
priate expression vector, no additional translational
control signals may be needed. However, in cases
5 where only a portion of the Flk-1 coding sequence is
inserted, exogenous translational control signals,
including the ATG initiation codon, must be provided.
Furthermore, the initiation codon must be in phase
with the reading frame of the Flk-1 coding sequence to
10 ensure translation of the entire insert. These
exogenous translational control signals and initiation
codons can be of a variety of origins, both natural
and synthetic. The efficiency of expression may be
enhanced by the inclusion of appropriate transcription
15 enhancer elements, transcription terminators, etc.
(see Bittner et al., 1987, Methods in Enzymol.
153:516-544).

In addition, a host cell strain may be chosen
which modulates the expression of the inserted
20 sequences, or modifies and processes the gene product
in the specific fashion desired. Such modifications
(e.g., glycosylation) and processing (e.g., cleavage)
of protein products may be important for the function
of the protein. Different host cells have charac-
25 teristic and specific mechanisms for the post-transla-
tional processing and modification of proteins.
Appropriate cells lines or host systems can be chosen
to ensure the correct modification and processing of
the foreign protein expressed. To this end,
30 eukaryotic host cells which possess the cellular
machinery for proper processing of the primary
transcript, glycosylation, and phosphorylation of the
gene product may be used. Such mammalian host cells
include but are not limited to CHO, VERO, BHK, HeLa,
35 COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the Flk-1 may be engineered. Rather than using expression vectors

5 which contain viral origins of replication, host cells can be transformed with the Flk-1 DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable

10 marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the

15 selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the Flk-1 on the

20 cell surface, and which respond to VEGF mediated signal transduction. Such engineered cell lines are particularly useful in screening VEGF analogs.

A number of selection systems may be used, including but not limited to the herpes simplex virus

25 thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be

30 employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl.

35 Acad. Sci. USA 78:1527); gpt, which confers resistance

to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and

5   hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows

10 cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO

15 (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

5.2.2.   IDENTIFICATION OF TRANSFECTANTS OR TRANSFORMANTS THAT EXPRESS THE Flk-1

20
        The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence

25 or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of Flk-1 mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

30   In the first approach, the presence of the Flk-1 coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the Flk-1 coding sequence, respectively,

35 or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity,

5 resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the Flk-1 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the Flk-1 coding

10 sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the Flk-1 sequence under the control of the same or different promoter used to control the expression of the Flk-1 coding sequence.

15 Expression of the marker in response to induction or selection indicates expression of the Flk-1 coding sequence.

In the third approach, transcriptional activity for the Flk-1 coding region can be assessed by

20 hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the Flk-1 coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for

25 hybridization to such probes.

In the fourth approach, the expression of the Flk-1 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays

30 and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active Flk-1 gene product. A number of assays can be used to detect receptor activity including but not limited to VEGF binding assays; and

35

VEGF biological assays using engineered cell lines as
the test substrate.

5.3. USES OF THE Flk-1 RECEPTOR
AND ENGINEERED CELL LINES

5

Angiogenesis, the growth of new blood capillary
vessels, is required for a number of physiological
processes ranging from wound healing, tissue and organ
regeneration, placental formation after pregnancy and

10 embryonic development. Abnormal proliferation of
blood vessels is an important component of a variety
of diseases such as rheumatoid arthritis,
retinopathies, and psoriasis. Angiogenesis is also an
important factor in the growth and metastatic activity

15 of solid tumors that rely on vascularization.
Therefore, inhibitors of angiogenesis may be used
therapeutically for the treatment of diseases
resulting from or accompanied by abnormal growth of
blood vessels and for treatments of malignancies

20 involving growth and spread of solid tumors.
In an embodiment of the invention the Flk-1
receptor and/or cell lines that express the Flk-1
receptor may be used to screen for antibodies,
peptides, organic molecules or other ligands that act

25 as agonists or antagonists of angiogenesis or
vasculogenesis mediated by the Flk-1 receptor. For
example, anti-Flk-1 antibodies capable of neutralizing
the activity of VEGF, may be used to inhibit Flk-1
function. Additionally, anti-Flk-1 antibodies which

30 mimic VEGF activity may be selected for uses in wound
healing. Alternatively, screening of peptide
libraries or organic compounds with recombinantly
expressed soluble Flk-1 protein or cell lines
expressing Flk-1 protein may be useful for

35

identification of therapeutic molecules that function
by inhibiting the biological activity of Flk-1.

In an embodiment of the invention, engineered
cell lines which express the entire Flk-1 coding
5   region or its ligand binding domain may be utilized to
screen and identify VEGF antagonists as well as
agonists. Synthetic compounds, natural products, and
other sources of potentially biologically active
materials can be screened in a number of ways. The
10 ability of a test compound to inhibit binding of VEGF
to Flk-1 may be measured using standard receptor
binding techniques, such as those described in Section
6.1.9., or using any of the compound screening assays
described in Section 5.3.2. and 6.1.14. The ability
15 of agents to prevent or mimic, the effect of VEGF
binding on signal transduction responses on Flk-1
expressing cells may be measured. For example,
responses such as activation of Flk-1 kinase activity,
modulation of second messenger production or changes
20 in cellular metabolism may be monitored. These assays
may be performed using conventional techniques
developed for these purposes.

The ability of a test compound to modulate signal
transduction through the VEGF-Flk-1 system may also be
25 measured *in vivo*, in models such as those described in
Section 6.1.12. and 6.1.13. The ability of agents to
prevent the effect of VEGF binding on signal
transduction responses of Flk-1 expressing cells may
be measured. For example, responses such as
30 inhibition of angiogenesis, inhibition of the
development of solid tumors, or reduction of solid
tumor size may be monitored.

35

5.3.1. SCREENING OF PEPTIDE LIBRARY WITH Flk-1 PROTEIN OR ENGINEERED CELL LINES

Random peptide libraries consisting of all
possible combinations of amino acids attached to a
solid phase support may be used to identify peptides
that are able to bind to the ligand binding site of a
given receptor or other functional domains of a
receptor such as kinase domains (Lam, K.S. et al.,
1991, Nature 354: 82-84). The screening of peptide
libraries may have therapeutic value in the discovery
of pharmaceutical agents that act to inhibit the
biological activity of receptors through their
interactions with the given receptor.

Identification of molecules that are able to bind
to the Flk-1 may be accomplished by screening a
peptide library with recombinant soluble Flk-1
protein. Methods for expression and purification of
Flk-1 are described in Section 5.2.1 and may be used
to express recombinant full length Flk-1 or fragments
of Flk-1 depending on the functional domains of
interest. For example, the kinase and extracellular
ligand binding domains of Flk-1 may be separately
expressed and used to screen peptide libraries.

To identify and isolate the peptide/solid phase
support that interacts and forms a complex with Flk-1,
it is necessary to label or "tag" the Flk-1 molecule.
The Flk-1 protein may be conjugated to enzymes such as
alkaline phosphatase or horseradish peroxidase or to
other reagents such as fluorescent labels which may
include fluorescein isothyiocynate (FITC),
phycoerythrin (PE) or rhodamine. Conjugation of any
given label, to Flk-1, may be performed using
techniques that are routine in the art.
Alternatively, Flk-1 expression vectors may be
engineered to express a chimeric Flk-1 protein

containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including labeling with enzymes, fluorescent dyes

5 or colored or magnetic beads.

The "tagged" Flk-1 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between Flk-1 and peptide species within the library. The library is

10 then washed to remove any unbound Flk-1 protein. If Flk-1 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing a substrates for either alkaline phosphatase or peroxidase, for example, 5-

15 bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diamnobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-Flk-1 complex changes color, and can be easily identified and isolated physically under a dissecting

20 microscope with a micromanipulator. If a fluorescent tagged Flk-1 molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric Flk-1 protein expressing a heterologous epitope has been used, detection of the peptide/Flk-1

25 complex may be accomplished by using a labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

In addition to using soluble Flk-1 molecules, in

30 another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with receptors that require the lipid domain of the

35 cell membrane to be functional. Methods for

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generating cell lines expressing Flk-1 are described in Sections 5.2.1. and 5.2.2. The cells used in this technique may be either live or fixed cells. The cells will be incubated with the random peptide

5 library and will bind to certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a

10 dissecting microscope.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into

15 liposomes where label or "tag" can be attached.

5.3.2. SCREENING OF ORGANIC COMPOUNDS WITH Flk-1 PROTEIN OR ENGINEERED CELL LINES

Cell lines that express Flk-1 may be used to

20 screen for molecules that modulate Flk-1 receptor activity or signal transduction. Such molecules may include small organic or inorganic compounds, or other molecules that modulate Flk-1 receptor activity or that promote or prevent the formation of Flk-1/VEGF

25 complex. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways.

The ability of a test molecule to interfere with VEGF-Flk-1 binding and/or Flk-1 receptor signal may be

30 measured using standard biochemical techniques. Other responses such as activation or suppression of catalytic activity, phosphorylation or dephosphorylation of other proteins, activation or modulation of second messenger production, changes in

35 cellular ion levels, association, dissociation or

translocation of signalling molecules, or
transcription or translation of specific genes may
also be monitored. These assays may be performed
using conventional techniques developed for these
5 purposes in the course of screening.

Ligand binding to its cellular receptor may, via
signal transduction pathways, affect a variety of
cellular processes. Cellular processes under the
control of the Flk-1/VEGF signalling pathway may
10 include, but are not limited to, normal cellular
functions, proliferation, differentiation, maintenance
of cell shape, and adhesion, in addition to abnormal
or potentially deleterious processes such as
unregulated cell proliferation, loss of contact
15 inhibition, blocking of differentiation or cell death.
The qualitative or quantitative observation and
measurement of any of the described cellular processes
by techniques known in the art may be advantageously
used as a means of scoring for signal transduction in
20 the course of screening.

Various embodiments are described below for
screening, identification and evaluation of compounds
that interact with the Flk-1 receptor, which compounds
may affect various cellular processes under the
25 control of the Flk/VEGF receptor signalling pathway.

The present invention includes a method for
identifying a compound which is capable of modulating
signal transduction, comprising:

(a)   contacting the compound with Flk-1, or a
30   functional derivative thereof, in pure or
          semi-pure form, in a membrane preparation,
          or in a whole live or fixed cell;

(b)   incubating the mixture of step (a) in the
          presence of VEGF, for an interval sufficient

35

for the compound to stimulate or inhibit the
signal transduction;

(c)   measuring the signal transduction;

(d) - comparing the signal transduction activity

5   to that of Flk-1, incubated without the
compound, thereby determining whether the
compound stimulates or inhibits signal
transduction.

Flk-1, or functional derivatives thereof, useful

10 in identifying compounds capable of modulating signal
transduction may have, for example, amino acid
deletions and/or insertions and/or substitutions as
long as they retain significant signal transducing
capacity. A functional derivative of Flk-1 may be

15 prepared from a naturally occurring or recombinantly
expressed Flk-1 by proteolytic cleavage followed by
conventional purification procedures known to those
skilled in the art. Alternatively, the functional
derivative may be produced by recombinant DNA

20 technology by expressing parts of Flk-1 which include
the functional domain in suitable cells. Functional
derivatives may also be chemically synthesized. Cells
expressing Flk-1 may be used as a source of Flk-1,
crude or purified, or in a membrane preparation, for

25 testing in these assays. Alternatively, whole live or
fixed cells may be used directly in those assays.

Flk-1 signal transduction activity may be
measured by standard biochemical techniques or by
monitoring the cellular processes controlled by the

30 signal. To assess modulation of kinase activity, the
test molecule is added to a reaction mixture
containing Flk-1 and a substrate test. To assess
modulation of kinase activity of the Flk-1 receptor,
the test molecule is added to a reaction mixture

35 containing the Flk-1 receptor. The kinase reaction is

then initiated with the addition of VEGF and ATP. An immunoassay is performed on the kinase reaction to detect the presence or absence of the phosphorylated tyrosine residues on the substrate or to detect

5   phosphorylated tyrosine residues on autophosphorylated Flk-1, and results are compared to those obtained for controls i.e., reaction mixtures not exposed to the test molecule. The immunoassay used to detect the phosphorylated substrate in the cell lysate or the in

10   vitro reaction mixture may be carried out with an anti-phosphotyrosine antibody.

The invention further provides for a method of screening compounds that, upon interacting with Flk-1, elicit or trigger a signal mimicking the action of

15   VEGF binding to the Flk-1 receptor. Signal transduction is mimicked if the cellular processes under the control of the signalling pathway are affected in a way similar to that caused by ligand binding. Such compounds may be naturally occurring or

20   synthetically produced molecules that activate the Flk-1 receptor.

The invention also includes a method whereby a molecule capable of binding to Flk-1 in a chemical or biological preparation may be identified comprising:

25   (a)   immobilizing Flk-1, or functional fragments thereof, to a solid phase matrix;

         (b)   contacting the chemical or biological preparation with the solid phase matrix produced in step (a), for an interval

30   sufficient to allow the compound to bind;

         (c)   washing away any unbound material from the solid phase matrix;

         (d)   detecting the presence of the compound bound to the solid phase,

35   thereby identifying the compound.

The above method may further include the step of:

    (e)   eluting the bound compound from the solid
            phase matrix, thereby isolating the
            compound.

The term "compound capable of binding to Flk-1"
refers to a naturally occurring or synthetically
produced molecule which interacts with Flk-1. Such a
compound may directly or indirectly modulate Flk-1
signal transduction and may include molecules that are
natively associated with the intracellular domain of
Flk-1 inside a cell. Examples of such compounds are
(i) a natural substrate of Flk-1; (ii) a naturally
occurring molecule which is part of the signalling
complex; and/or a naturally occurring signalling
molecule produced by other cell types.

5.3.3. ANTIBODY PRODUCTION AND SCREENING

Various procedures known in the art may be used
for the production of antibodies to epitopes of the
recombinantly produced Flk-1 receptor. Such
antibodies include but are not limited to polyclonal,
monoclonal, chimeric, single chain, Fab fragments and
fragments produced by an Fab expression library.
Neutralizing antibodies i.e., those which compete for
the VEGF binding site of the receptor are especially
preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind Flk-1 may be
radioactively labeled allowing one to follow their
location and distribution in the body after injection.
Radioactivity tagged antibodies may be used as a non-
invasive diagnostic tool for imaging *de novo*
vascularization associated with a number of diseases
including rheumatoid arthritis, macular degeneration,
and formation of tumors and metastases.

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity Flk-1 specific monoclonal antibodies may be covalently complexed to bacterial or plant toxins, such as diptheria toxin, abrin or ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin to the antibody. The hybrid antibodies may be used to specifically eliminate Flk-1 expressing endothelial cells.

For the production of antibodies, various host animals may be immunized by injection with the Flk-1 protein including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies to Flk-1 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma tech- nique (Cole et al., 1985, Monoclonal Antibodies and

Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In
addition, techniques developed for the production of
"chimeric antibodies" (Morrison et al., 1984, Proc.
Natl. Acad. Sci., 81:6851-6855; Neuberger et al.,
1984, Nature, 312:604-608; Takeda et al., 1985,
Nature, 314:452-454) by splicing the genes from a
mouse antibody molecule of appropriate antigen
specificity together with genes from a human antibody
molecule of appropriate biological activity can be
used. Alternatively, techniques described for the
production of single chain antibodies (U.S. Patent
4,946,778) can be adapted to produce Flk-1-specific
single chain antibodies.

Antibody fragments which contain specific binding
sites of Flk-1 may be generated by known techniques.
For example, such fragments include but are not
limited to: the $F(ab')_2$ fragments which can be produced
by pepsin digestion of the antibody molecule and the
Fab fragments which can be generated by reducing the
disulfide bridges of the $F(ab')_2$ fragments. Alterna-
tively, Fab expression libraries may be constructed
(Huse et al., 1989, Science, 246:1275-1281) to allow
rapid and easy identification of monoclonal Fab
fragments with the desired specificity to Flk-1.

5.4. USES OF Flk-1 CODING SEQUENCE
The Flk-1 coding sequence may be used for
diagnostic purposes for detection of Flk-1 expression.
Included in the scope of the invention are
oligoribonucleotide sequences, that include antisense
RNA and DNA molecules and ribozymes that function to
inhibit translation of Flk-1. In addition, mutated
forms of Flk-1, having a dominant negative effect, may
be expressed in targeted cell populations to inhibit

the activity of endogenously expressed wild-type Flk-1.

5.4.1. USE OF Flk-1 CODING SEQUENCE IN DIAGNOSTICS AND THERAPEUTICS

The Flk-1 DNA may have a number of uses for the diagnosis of diseases resulting from aberrant expression of Flk-1. For example, the Flk-1 DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of Flk-1 expression; e.g., Southern or Northern analysis, including in situ hybridization assays.

The Flk-1 cDNA may be used as a probe to detect the expression of the Flk-1 mRNA. In a specific example described herein, the expression of Flk-1 mRNA in mouse embryos of different developmental stages was analyzed. Northern blot analysis indicated abundant expression of a major 5.5 kb mRNA between day 9.5 and day 18.5, with apparent decline towards the end of gestation (FIG. 2A). In post-natal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain RNA (FIG.2B), suggesting a role for Flk-1 in endothelial cell proliferation.

To obtain more detailed information about the expression of Flk-1 during embryonic development and during the early stages of vascular development in situ hybridization experiments were performed as described in Section 6.1.4. In situ hybridizations demonstrated that Flk-1 expression *in vivo* during embryonic mouse development is largely restricted to endothelial cells and their precursors (FIGS. 3A, 3B, 3C and FIGS. 4A, 4B, 4C, 4D and 4E). Flk-1 is expressed in endothelial cells during physiological processes that are characterized by endothelial cell proliferation and the temporal and spatial expression

pattern found in the embryonic brain correlate precisely with the development of the neural vascular system as described by Bar (1980). Vascular sprouts originating in the perineural plexus grow radially
5 into the neuroectoderm and branch there and these sprouts were found to express high amounts of Flk-1 mRNA (FIGS. 5A, 5B, 5C and 5D). In the early postnatal stages, endothelial cell proliferation is still evident and Flk-1 is expressed, whereas in the
10 adult organism, after completion of the vascularization process, the decline in endothelial cell proliferation parallels a decrease in Flk-1 expression.

       Also within the scope of the invention are oligo-
15 ribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of Flk-1 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and
20 preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the Flk-1 nucleotide sequence, are preferred.

25   Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleav-
30 age. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of Flk-1 RNA sequences.

       Specific ribozyme cleavage sites within any
35 potential RNA target are initially identified by

scanning the target molecule for ribozyme cleavage
sites which include the following sequences, GUA, GUU
and GUC. Once identified, short RNA sequences of
between 15 and 20 ribonucleotides corresponding to the
5 region of the target gene containing the cleavage site
may be evaluated for predicted structural features
such as secondary structure that may render the oligo-
nucleotide sequence unsuitable. The suitability of
candidate targets may also be evaluated by testing
10 their accessibility to hybridization with complemen-
tary oligonucleotides, using ribonuclease protection
assays.

    Both anti-sense RNA and DNA molecules and
ribozymes of the invention may be prepared by any
15 method known in the art for the synthesis of RNA
molecules. These include techniques for chemically
synthesizing oligodeoxyribonucleotides well known in
the art such as for example solid phase
phosphoramidite chemical synthesis. Alternatively,
20 RNA molecules may be generated by *in vitro* and *in vivo*
transcription of DNA sequences encoding the antisense
RNA molecule. Such DNA sequences may be incorporated
into a wide variety of vectors which incorporate
suitable RNA polymerase promoters such as the T7 or
25 SP6 polymerase promoters. Alternatively, antisense
cDNA constructs that synthesize antisense RNA
constitutively or inducibly, depending on the promoter
used, can be introduced stably into cell lines.

    Various modifications to the DNA molecules may be
30 introduced as a means of increasing intracellular
stability and half-life. Possible modifications
include but are not limited to the addition of
flanking sequences of ribo- or deoxy- nucleotides to
the 5' and/or 3' ends of the molecule or the use of
35 phosphorothioate or 2' O-methyl rather than phospho-

diesterase linkages within the oligodeoxyribonucleo-
tide backbone.

5.4.2. USE OF DOMINANT NEGATIVE Flk-1 MUTANTS IN GENE THERAPY

Receptor dimerization induced by ligands, is
thought to provide an allosteric regulatory signal
that functions to couple ligand binding to stimulation
of kinase activity. Defective receptors can function
as dominant negative mutations by suppressing the
activation and response of normal receptors through
formation of heterodimers with wild type receptors
wherein such heterodimers are signalling incompetent.
Defective receptors can be engineered into recombinant
viral vectors and used in gene therapy in individuals
that inappropriately express Flk-1.

The capability of Flk-1 TM to form signalling
incompetent heterodimers with the 180 kD wild type
Flk-1 is demonstrated in Section 6.1.12. The
dominant-negative potential of Flk-1 TM used in gene
therapy may be measured by examining its influence on
the Flk-1/ VEGF mitogenic response or by measurement
of suppression of Flk-1 transforming activity.

In an embodiment of the invention, mutant forms
of the Flk-1 molecule having a dominant negative
effect may be identified by expression in selected
cells. Deletion or missense mutants of Flk-1 that
retain the ability to form dimers with wild type Flk-1
protein but cannot function in signal transduction may
be used to inhibit the biological activity of the
endogenous wild type Flk-1. For example, the
cytoplasmic kinase domain of Flk-1 may be deleted
resulting in a truncated Flk-1 molecule that is still
able to undergo dimerization with endogenous wild type
receptors but unable to transduce a signal.

Abnormal proliferation of blood vessels is an important component of a variety of pathogenic disorders such as rheumatoid arthritis, retinopathies and psoriasis. Uncontrolled angiogenesis is also an

5   important factor in the growth and metastases of solid tumors. Recombinant viruses may be engineered to express dominant negative forms of Flk-1 which may be used to inhibit the activity of the wild type endogenous Flk-1. These viruses may be used

10   therapeutically for treatment of diseases resulting from aberrant expression or activity of Flk-1.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used

15   for delivery of recombinant Flk-1 into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing Flk-1 coding sequence. See, for example, the techniques described

20   in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant

25   Flk-1 molecules can be reconstituted into liposomes for delivery to target cells.

In a specific embodiment of the invention, a deletion mutant of the Flk-1 receptor was engineered into a recombinant retroviral vector. Two clonal

30   isolates of Flk-1 TM, designated pLXSN Flk-1 TM cl.1 and pLXSN Flk-1 TM cl.3, contain a truncated Flk-1 receptor containing Flk-1 amino acids 1 through 806 but lacking the 561 COOH-terminal amino acids of the intracellular kinase domain. These isolates retain

35   transmembrane domain sequences and 23 residues of the

cytoplasmic domain. To obtain virus producing cell lines, PA37 cells were transfected with the recombinant vectors and, subsequently, conditioned media containing virus were used to infect GPE cells.

5   To test whether expression of signaling-defective mutants inhibits endogenous Flk-1 receptor activity, C6 rat gliobastoma cells (tumor cells) and mouse cells producing the recombinant retroviruses were mixed and injected subcutaneously into nude mice. Normally,

10   injection of tumor cells into nude mice would result in proliferation of the tumor cells and vascularization of the resulting tumor mass. Since Flk-1 is believed to be essential for formation of blood vessels, blocking Flk-1 activity by expression

15   of a truncated receptor, might function to inhibit vascularization of the developing tumor and, thereby, inhibit its growth. As illustrated in Figures 13 and 14, coinjection of virus producing cells, expressing a truncated Flk-1 receptor, significantly inhibits the

20   growth of the tumor as compared to controls receiving only tumor cells.

As illustrated in Figure 15, a similar inhibitory effect on C6 glioma tumor growth was also observed when truncated Flk-1 receptor virus particle-

25   containing producer cells were injected five days after implantation of 10^6 tumor cells, indicating that even established tumors may be suppressed by Flk-1 dominant-negative action. For glioblastoma, a tumor with generally poor prognosis and resistance to all

30   available therapies, retrovirus-mediated gene therapy may be advantageous, since non-mitotic brain tissues such as neurons, glia and quiescent endothelial cells would not be infected. Glioblastoma multiforme is the most common and most malignant tumor of astrocytic

35   origin in human adults and accounts for more than half

of all primary brain tumors (See, for example, <u>Cecil</u>
<u>Textbook of Medicine</u>, Wyngaarden, Smith, Bennett (eds)
WB Saunders, p.2220 **(1992).**

5   5.5. <u>USE OF Flk-1 RECEPTOR OR LIGANDS</u>
       Receptor/ligand interaction between Flk-1 and
VEGF is believed to play an important role in the
signalling system during vascularization and
angiogenesis. Abnormal proliferation of blood vessels
10 is an important component of a number of diseases.
       Expression of Flk-1 RNA correlates with the
development of the brain and with endothelial cell
proliferation suggesting that Flk-1 might be a
receptor involved in mediation of signaling events in
15 the neural vascularization process. VEGF has been
shown to be a mitogenic growth factor known to act
exclusively on endothelial cells (Ferrara, N. and
Henzel, W.J., 1989, Biochem. Biophys. Res. Comm.
161:851-858). Cross-linking and ligand binding
20 experiments were performed, as described in Sections
6.1.9 and 6.1.10 respectively, to determine whether
VEGF is a ligand for Flk-1. The results indicate that
Flk-1 is an authentic high affinity VEGF receptor
(FIGS. 9A and 9B).
25   In one embodiment of the invention, ligands for
Flk-1, the Flk-1 receptor itself, or a fragment
containing its VEGF binding site, could be
administered <u>in</u> <u>vivo</u> to modulate angiogenesis and/or
vasculogenesis. For example, administration of the
30 Flk-1 receptor or a fragment containing the VEGF
binding site, could competitively bind to VEGF and
inhibit its interaction with the native Flk-1 receptor
<u>in</u> <u>vivo</u> to inhibit angiogenesis and/or vasculogenesis.
Alternatively, ligands for Flk-1, including anti-Flk-1
35 antibodies or fragments thereof, may be used to

modulate angiogenesis and/or vasculogenesis. Agonists of VEGF activity may be used to promote wound healing whereas antagonists of VEGF activity may be used to inhibit tumor growth.

5   The particular peptides, proteins, organic compounds or antibodies that modulate Flk-1 receptor signal transduction can be administered to a patient either by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or

10   excipient(s).

Depending on the specific conditions being treated, these agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in

15   "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. Suitable routes may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary

20   injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, or, in the case of solid tumors, directly injected into a solid tumor. For injection, the agents of the invention may be

25   formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in

30   the formulation. Such penetrants are generally known in the art.

The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration.

35   Such carriers enable the compounds of the invention to

be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Pharmaceutical compositions suitable for use in
5   the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of
10 the detailed disclosure provided herein.

In addition to the active ingredients these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing
15 of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present
20 invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

25   Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable
30 lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such
35 as sodium carboxymethyl cellulose, sorbitol, or

dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

5   Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or

10   dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl

15   cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium

20   alginate.

        Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene

25   glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

30   Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients

35   in admixture with filler such as lactose, binders such

as starches, and/or lubricants such as talc or
magnesium stearate and, optionally, stabilizers. In
soft capsules, the active compounds may be dissolved
or suspended in suitable liquids, such as fatty oils,
5 liquid paraffin, or liquid polyethylene glycols. In
addition, stabilizers may be added.

Compositions comprising a compound of the
invention formulated in a compatible pharmaceutical
carrier may be prepared, placed in an appropriate
10 container, and labelled for treatment of an indicated
condition. Suitable conditions indicated on the label
may include treatment of a tumor, such as a glioma or
glioblastoma; and inhibition of angiogenesis.

A preferred pharmaceutical carrier for
15 hydrophobic compounds of the invention is a cosolvent
system comprising benzyl alcohol, a nonpolar
surfactant, a water-miscible organic polymer, and an
aqueous phase. A preferred cosolvent system is the
VPD co-solvent system. VPD is a solution of 3% w/v
20 benzyl alcohol, 8% w/v of the nonpolar surfactant
polysorbate 80, and 65% w/v polyethylene glycol 300,
made up to volume in absolute ethanol. The VPD co-
solvent system (VPD:5W) consists of VPD diluted 1:1
with a 5% dextrose in water solution. This co-solvent
25 system dissolves hydrophobic compounds well, and
itself produces low toxicity upon systemic
administration. Naturally, the proportions of a co-
solvent system may be varied considerably without
destroying its solubility and toxicity
30 characteristics. Furthermore, the identity of the co-
solvent components may be varied: for example, other
low-toxicity nonpolar surfactants may be used instead
of polysorbate 80; the fraction size of polyethylene
glycol may be varied; other biocompatible polymers may
35 replace polyethylene glycol, e.g. polyvinyl

pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed.

5 Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as DMSO also may be employed, although usually at the cost of greater toxicity.

10 The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose

15 derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the Flk-1 receptor modulating compounds of the invention may be provided as salts with pharmaceutically compatible counterions.

20 Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding

25 free base forms.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to

30 achieve a circulating concentration range that includes the IC50 as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the PTP activity). Such information can be used to more accurately determine

35 useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds

5 can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population).

10 The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can

15 be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the

20 dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The

25 Pharmacological Basis of Therapeutics", Ch. 1 p1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the Flk-1 receptor-inhibitory effects. Usual patient dosages

30 for systemic administration range from 1 - 2000 mg/day, commonly from 1 - 250 mg/day, and typically from 10 -150 mg/day. Stated in terms of patient body weight, usual dosages range from 0.02 - 25 mg/kg/day, commonly from 0.02 - 3 mg/kg/day, typically from 0.2 -

35 1.5 mg/kg/day. Stated in terms of patient body

surface areas, usual dosages range from 0.5 - 1200 mg/m²/day, commonly from 0.5 - 150 mg/m²/day, typically from 5 - 100 mg/m²/day.

5 Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the Flk-1 receptor-inhibitory effects. Usual average plasma levels should be maintained within 50 - 5000 μg/ml, commonly 50 - 1000 μg/ml, and typically 100 - 500

10 μg/ml

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a tumor, often in a depot or sustained release formulation.

15 Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

20 In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

6. EXAMPLE: CLONING AND EXPRESSION PATTERNS
   OF Flk-1, A HIGH AFFINITY RECEPTOR FOR VEGF

25 The subsection below describes the cloning and characterization of the Flk-1 cDNA clone. Northern blot and *in situ* hybridization analyses indicate that Flk-1 is expressed in endothelial cells. Cross-

30 linking and ligand binding experiments further indicate that Flk-1 is a high affinity receptor for VEGF.

35

6.1. MATERIALS AND METHODS

6.1.1. cDNA CLONING OF Flk-1

DNA extracted from λgt10 cDNA library of day 8.5
mouse embryos (Fahrner et al., 1987, EMBO. J. 6:1497-
5   1508) was used as template for polymerase chain
reaction (PCR; Saiki, R.K. et al., 1985 Science
230:1350-1354). In an independent approach cDNA of
capillary endothelial cells that had been isolated
from the brain of postnatal day 4-8 mice was used for
10 amplification (Risau, W., 1990 In: development of the
Vascular System. Issues Biomed. Basel Karger 58-68
and Schnürch et al., unpublished) Degenerated primers
were designed on the basis of high amino acid
homologies within the kinase domain shared by all RTKs
15 (Wilks, A.F., 1989, Proc. Natl. Acad. Sci. U.S.A.
86:1603-1607).

Full length cDNA clones of Flk-1 were isolated
from another day 8.5 mouse embryo cDNA library, which
had been prepared according to the method of Okayama
20 and Berg (1983), and a day 11.5 mouse embryo λgt11
library (Clonetech) using the ^{32}P-labeled (Feinberg,
A.P. and Vogelstein, B. 1983 Anal. Biochem. 132:6-13)
210-bp PCR fragment.

25   6.1.2. MOUSE EMBRYOS

Balb/c mice were mated overnight and the morning
of vaginal plug detection was defined as 1/2 day of
gestation. For Northern blot analysis the frozen
embryos were homogenized in 5 M guanidinium thiocya-
30 nate and RNA was isolated as described (Ullrich, A. et
al., 1985, Nature 313:756-761). For in situ
hybridization, the embryos were embedded in Tissue-Tek
(Miles), frozen on the surface of liquid nitrogen and
stored at -70C prior to use.

35

6.1.3. PREPARATION OF PROBES

The 5'-located 2619 bp of the receptor cDNA were subcloned in the pGem3Z vector (Promega) as an EcoR1/ BamH1 fragment. The probe for Northern blot hybrid-

5 ization was prepared by labelling the cDNA fragment with $\alpha-^{32}PdATP$ (Amersham) by random hexanucleotide priming (Boehringer; Feinberg, A.P. and Vogelstein, B., 1983 Anal. Biochem. 132:6-13).

For *in situ* hybridization a single-strand

10 antisense DNA probe was prepared as described by Schnürch and Risau (Development, 1991 111:1143-54). The plasmid was linearized at the 3' end of the cDNA and a sense transcript was synthesized using SP6 RNA polymerase (Boehringer). The DNA was degraded using

15 DNAase (RNAase free preparation, Boehringer Mannheim). With the transcript, a random-primed cDNA synthesis with a $\alpha-^{35}S$ dATP (Amersham) was performed by reverse transcription with MMLV reverse transcriptase (BRL). To obtain small cDNA fragments of about 100 bp in

20 average suitable for *in situ* hybridization, a high excess of primer was used. Subsequently the RNA transcript was partially hydrolyzed in 100 mM NaOH for 20 minutes at 70°C, and the probe was neutralized with the same amount of HC1 and purified with a Sephadex

25 C50 column. After ethanol precipitation the probe was dissolved at a final specific activity of $5x10^5$ cpm. For control hybridization a sense probe was prepared with the same method.

30   ### 6.1.4. RNA EXTRACTION AND
                 NORTHERN ANALYSIS

Total cytoplasmic RNA was isolated according to the acidic phenol-method of Chromczynski and Sacchi (1987). Poly(A⁺) RNA aliquots were electrophoresed in

35 1.2% agarose formaldehyde (Sambrook, J. et al., 1989 Molecular Cloning: A Laboratory Manual 2nd ed. Cold

Spring Harbor Laboratory Press) gels and transferred
to nitrocellulose membranes (Schleicher & Schuell),
Hybridizations were performed overnight in 50%
formamide, 5 x SSC (750mM sodium chloride, 75mM sodium

5   citrate), 5 x Denhardt's (0.1% Ficoll 400, 0.1%
polyvinylpyrrolidone, 0.1% BSA) and -0.5% SDS at 42°C
with 1-3x10^6 cpm-ml-^{1} of ^{32}P-Random primed DNA probe,
followed by high stringency washes in 0.2 x SSC, 0.5%
SDS at 52°C. The filters were exposed for 4 to 8

10 days.

6.1.5. *IN SITU* HYBRIDIZATION

Subcloning postfixation and hybridization was
essentially performed according to Hogan et al.

15 (1986). 10 μm thick sections were cut at -18°C on a
Leitz cryostat. For prehybridization treatment no
incubation with 0.2M HCl for removing the basic
proteins was performed. Sections were incubated with
the ^{35}S-cDNA probe (5x10^{4}cpm/μl) at 52°C in a buffer

20 containing 50% formamide, 300 mM NaCl, 10 mM Tris-HCl,
10 mM NaPO$_4$ (pH 6.8), 5 mM EDTA, 0.02% Ficoll 400,
0.01% polyvinylpyrrolidone, 0.02% BSA, 10 m /ml yeast
RNA, 10% dextran sulfate, and 10 mM NaCl, 10 mM Tris-
HCl, 10 mM NaPO$_4$ (pH 6.8), 5 mM EDTA, 10 Mm DTT at

25 52°C). For autoradiography, slides were coated with
Kodak NTB2 film emulsion and exposed for eight days.
After developing, the sections were counterstained and
toluidine blue or May-Grinwald.

30   ### 6.1.6. PREPARATION OF ANTISERA

The 3' primed EcoRV/HindII fragment comprising
the 128 C-terminal amino acids of Flk-1 was subcloned
in the fusion protein expression vector pGEX3X (Smith,
D.B. and Johnson, K.S., 1990 Gene. 67:31-40;

35 Pharmacia). The fusion protein was purified as

described and used for immunizing rabbits. After the second boost the rabbits were bled and the antiserum was used for immunoprecipitation.

6.1.7. TRANSIENT EXPRESSION OF Flk-1 IN COS-1 CELLS

Transfection of COS-1 cells was performed essentially as described by Chen and Okayama (1987 Mol. Cell. Biol. 7:2745-2752) and Gorman et al. (1989 Virology 171:377-385). Briefly, cells were seeded to a density of 1.0×10^6 per 10-cm dish and incubated overnight in DMEM containing 10% fetal calf serum (Gibco). 20 μg of receptor cDNA cloned into a cytomegalovirus promotor driven expression vector was mixed in 0.5 ml of 0.25 M $CaCa_2$, 0.5 ml of 2 x BBS (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM BES, pH 6.96 and incubated for 30 min at room temperature. The calcium phosphate/DNA solution was then added to the cells, swirled gently, and incubated for 18 hours at 37°C under 3% CO_2. For ligand binding experiments, the cells were removed from the plate and treated as described below.

To obtain VEGF conditioned media, cells were transfected in 15-cm dishes. Media was collected after 48 h and VEGF was partially purified by affinity chromatography using heparin High Trap TM columns (Pharmacia) and concentrated by ultrafiltration (Ferrara, N. and Henzel, W.J. 1989 Biochem. Biophys. Res. Comm. 161:851-858). The concentration of VEGF was determined by a ligand competition assay with bovine aortic endothelial cells.

For autophosphorylation assays, cells were seeded in 6-well dishes (2×10^5 cells per well), transfected as described above, and starved for 24 h in DMEM containing 0.5% fetal calf serum. The cells were then

treated with 500 pM VEGF for 10 min. at 37°C or left
untreated and were subsequently lysed as described by
Kris et al. (1985). Flk-1 was immunoprecipitated with
an antiserum raised in rabbits against the C-terminus

5 of the receptor. The immunoprecipitates were sepa-
rated on a 7.5% SDS polyacrylamide gel, transferred to
nitrocellulose, and incubated with a mouse monoclonal
antibody directed against phosphotyrosine (5E2;
Fendly, B.M. et al., 1990 Cancer Research 50:1550-

10 1558). Protein bands were visualized using
horseradish peroxidase coupled goat anti-mouse
antibody and the ECL™ (Amersham) detection system.

6.1.8. RADIOIODINATION OF VEGF

15   Recombinant human VEGF (5 µg; generously provided
by Dr. H. Weich) was dissolved in 110 µl sodium
phosphate buffer pH 76, and iodinated by the procedure
of Hunter and Greenwood (1962). The reaction products
were separated from the labeled protein by passage

20 over a sephadex G50 column, pre-equilibrated with
phosphate buffered saline (PBS) containing 0.7% bovine
serum albumin (BSA), and aliquots of the collected
fractions were counted before and after precipitation
with 20% trichloracetic acid. The purity of the

25 iodinated product was estimated to be superior to 90%,
as determined by gel electrophoresis, and the specific
activity was 77000 cpm/ng. The bioactivity of the
iodinated VEGF was confirmed by comparison with the
bioactivities of native VEGF using the tissue factor

30 introduction assay described by Clauss, M. et al.
(1990 J. Exp. Med. 172:1535-1545).

6.1.9. CROSSLINKING OF VEGF TO Flk-1

COS-1 cells transiently expressing Flk-1 and

35 untransfected COS-1 cells were incubated with 200 pM

^{125}I-VEGF at 4°C overnight, then washed twice with PBS
and exposed to 0.5 mM disuccinimidyl suberate (DSS) in
PBS for 1 h at 4°C. The cells were lysed, Flk-1
immunoprecipitated, and analyzed by electrophoresis on
5 a 7% polyacrylamide gel followed by autoradiography.

6.1.10. VEGF BINDING

Ligand binding experiments were performed as
described previously (Schumacher, R. et al., 1991, J.
10 Biol. Chem. 266:19288-19295), COS-1 cells were grown
in a 15-cm culture dish in DMEM for 48h after
transfection. Cells were then washed carefully with
PBS and incubated with 5 ml of 25 mM EDTA in PBS for
10 min. Cells were then removed from the plate,
15 washed once with binding buffer (DMEM, 25 mM HEPES, pH
7.5, 0.15% gelatin) and resuspended in 5 ml of binding
buffer to determine the cell number. In a total
volume of 500 μl this cell suspension was incubated
for 90 min at 15°C with 10 pM ^{125}I-VEGF, and increasing
20 concentration of unlabeled ligand (from 0 to 7 x 10^{-9}),
which was partially purified from conditioned media of
COS-1 cells transiently expressing VEGF (164 amino
acid form; Breier et al., Development vol. 114 (2) pp.
521-532 (1992). Leung et al., (Science vol. 246 pp.
25 1306-9 (1989) disclose cDNA clones for bovine and
human VEGF). After incubation, cells were washed
with PBS 0.1% PBS in the cold. Free ligand was
removed by repeated centrifugation and resuspension in
binding buffer. Finally, the ^{125}I radioactivity bound
30 to the cells were determined in a gamma counter
(Riastar). Data obtained were analyzed by the method
of Munson, P.J. and Rodbard, D. (1980 Anal. Biochem.
107:220-235).

35

6.1.11. RETROVIRAL VECTORS ENCODING
TRANSDOMINANT-NEGATIVE MUTANTS
OF Flk-1

Recombinant retroviral vectors were constructed
that contained the coding region for amino acids 1
through 806 of the Flk-1 receptor (pLX Flk-1 TM cl.1
and pLX Flk-1 TM cl.3, Figure 12A). A recombinant
virus containing the 541 N-terminal amino acids of the
CSF-1 receptor/c-fms (pNTK cfms TM cl.7, Figure 12B)
was used as a control.

pLXSN Flk-1 TM was obtained by ligating the 5'-
located 2619 bp of the Flk-1 cDNA encoding amino acids
1 to 806 as a ClaI/BamHI fragment to a BglII/HpaI
linker, thereby designing a stop-codon 23 amino acids
following the transmembrane region (5' GTC ATG GAT CTT
CGT TAA 3'). In a second step, the ClaI/HpaI fragment
was subcloned into the ClaI/HpaI site of the pLXSN
vector. Stable GP+E-86 cell lines producing ecotropic
retroviruses expressing the wild type and mutated
receptor constructs were generated as described by
Redemann et al. (Mol. Cell Biol. vol 12, p. 491-498
(1992)).

For generation of pNTK c-fms TM, a stop codon was
introduced behind amino acids 541 downstream from the
transmembrane region of the c-fms cDNA using the
oligonucleotide 5' TTG TAC AAG TAT AAG TAG TAG CCC AGG
TAC CAG 3'. The mutated receptor was subcloned in the
retroviral expression vector pNTK2 (Stewart et al.,
EMBO J., 6, 383-388 (1987). Stable GP+E-86 cell lines
were obtained as described above.

6.1.12. THE CAPABILITY OF FLK-1 TM TO FORM
SIGNALLING-INCOMPETENT HETERODIMERS

The capability of Flk-1 TM to form signalling
incompetent heterodimers with the 180 kD wild type
Flk-1 was demonstrated by coprecipitation of the

truncated 130 kD receptor mutant with an antibody
against the C-terminus of the intact receptor from
lysates of COS cells transiently expressing both
forms. Since the antibody could not recognize Flk-1
5 TM, coprecipitation was a consequence of
heterdimerization.

　　　To test the capability of Flk-1 TM to form
signalling incompetent heterodimers with the wild-type
Flk-1 *in vivo*, C6 gliobastoma tumor cells, available
10 from the ATCC, accession number CCL 107, were
implanted into nude mice either alone or coimplanted
with virus producing cells. Injected cell numbers for
the two sets of experiments are indicated below.
Beginning at the time when the first tumors appeared,
15 tumor volumes were measured every 2 to 3 days to
obtain a growth curve. The results are discussed in
Section 6.2.6 and shown in Figures 12 and 13.

Experiment No. 1

20

Number of Mice	Number of C6 Cells	Virus-Producer Cell Line	Number of Virus-Cells
4	5×10^5	pLXSN Flk-1 TM cl.3	1×10^7
4	5×10^5	None	0
4	5×10^5	pNTK cfms TM cl.7	5×10^6

25

Experiment No. 2

Number of Mice	Number of C6 Cells	Virus-Producer Cell Line	Number of Virus-Cells
4	2×10^6	pLXSN Flk-1 TM cl.1	2×10^7
4	2×10^6	pLXSN Flk-1 TM cl.3	2×10^7
4	2×10^6	None	0
4	2×10^6	pNTK cfms TM cl.7	2×10^7

30

35

In another experiment, the same experimental
conditions were performed except that the virus
producing cells were injected five days after
implantation of 10^6 tumor cells.

5   In another experiment, co-implantation of C6
glioblastoma cells was with different relative amounts
of retroviral cells producing comparable titers (1×10^6
cfu/ml) of recombinant retrovirus. The effect of the
inhibition of tumor growth was dose-dependent, with

10   maximum achieved when the virus-producing cells were
in 20-fold excess over the tumor cells. To confirm
that the inhibition of the C6 glioblastoma growth was
caused by dominant-negative action of the retrovirally
expressed constructs on endothelial cells, the tumors

15   were resected and analyzed. Comparison of the whole
mount specimens revealed striking differences:
whereas the control tumors exhibited a reddish
surface, as expected for well-vascularized tissue, the
inhibited cell implants were very pale. Histological

20   staining of frozen sections revealed that the control
tumors consisted of a homogenous mass of viable cells.
Only very few and small necroses could be detected.
In contrast, the much smaller, growth-inhibited tumor
cell implants had an onion-like histological

25   appearance, which was characterized by different
tissue layers: a large, central necrosis was
surrounded by a dense layer of viable tumor cells.
Invasion of this tumor had not progressed, as
evidenced by the presence of natural structures of the

30   skin, such as the muscular cell layer.
     The distribution of capillaries and blood vessels
in the tissue specimens was determined by incubating
frozen tissue sections with a rat monoclonal antibody
specific for the endothelial cell adhesion antigen

35   PECAM (De Vries et al., _Science_ vol. 255, pp. 989-991

(1992)). While the tumors coimplanted with the
control virus-producing cells displayed the pattern of
capillaries and vessels expected for well-vascularized
tissue, the growth-inhibited tumor cell implant

5 exhibited a large central tumor cell necrosis, which
was surrounded by a layer of viable tumor cells
lacking blood vessels or capillaries. The tumor cells
in this layer showed a higher cell density than the
control tumor suggesting a significant reduction in

10 tumor-induced edema formation. Since VEGF appears to
induce vascular permeability in vivo, and was
therefore also designated vascular permeability
factor, inhibition of VEGF/Flk-1 interaction may
inhibit tumor associated edema formation.

15   Flk-1 expression in proliferating endothelial
cells of the tumor was confirmed by *in situ*
hybridization of tissue sections with a ^{35}S-labeled
Flk-1 specific antisense cDNA probe and displayed the
same distribution as immunostaining with endothelial

20 cell-specific antibodies, indicating that
proliferating endothelial cells expressed Flk-1. *In
situ* hybridization with a neomycin resistance gene
(neo^r) antisense probe confirmed the presence of
retroviral sequences. The entire Flk-1 dominant-

25 negative-inhibited tumor consisting of the retrovirus-
producing and infected cells was neo^r-positive, a
result that exactly matched that obtained with a Flk-1
specific probe. The morphology of tumors that had
been coimplanted with control virus-producing cell was

30 very similar, but the virus-producing cells were
extensively infiltrated by infected tumor cells. In
these tumors, which contained many capillaries and
blood vessels, neo^r-positive signals were also found in
endothelial cells.

35

C6 gliomas exhibit morphological characteristics
of human glioblastoma multiforme such as necroses with
palisading cells, a high degree of vascularization,
and a similar expression pattern of VEGF and its
5   receptors making this model an excellent tool to study
anti-angiogenic therapy (Plate et al., Cancer Research
vol. 53, pp. 5822-5827 (1993)).

6.1.13. INTRACEREBRAL GRAFTING OF GLIOMA CELLS

10   To test the capability of Flk-1 TM to form
signalling incompetent heterodimers with the wild type
Flk-1 *in vivo*, C6 glioma cells were transplanted
intracranially into syngeneic rats with co-injection
of a retrovirus-producing cell line.
15   To transplant glioma cells intracranially into
rats, the rats (bodyweight 160-180 g) were
anesthetized by *i.p.* injection of 100 mg/kg Ketamin
(Ketaset^R) plus 5 mg/kg (Xylazine (Rompun^R). The
dosage is dependent on the rat strain and should be
20   determined before the experiments. Preanesthesia with
isoflurane facilitates *i.p.* injection and onset of
anesthesia. Approximately 3-10 minutes after *i.p.*
injection, animals no longer respond to pain. If
analgesia is not complete after 10 minutes, additional
25   dose of 50 mg/kg Ketanest plus 2.5 mg/kg Rompun *i.m.*
(50% of the initial *i.p.* dose) should be injected.
The animals were adjusted in a commercially available
small animal stereotactic apparatus. The skin was
cleaned with alcohol and a median incision
30   (approximately 1 cm in length) was performed over the
skull using a sterile surgical blade. The skin was
then slightly disattached from the skull. A burrhole
was made using a dental driller on the right side of
the hemisphere (coordinates: 2 mm lateral and 1 mm
35   anterior to the bregma). Care was taken not to

disrupt the meninges or to damage the brain (check via microscope). A hamilton syringe was placed in the burrhole at the level of the arachnoidea and then slowly lowered until the tip is 3 mm deep in the brain
5 structure (the target point is the cauda-putamen, lateral to the frontal horn of the lateral ventricle). Two to twenty microliters of cells (depending of the amount of cells one wishes to graft) were slowly injected. The maximum volume rats tolerate is 20-25
10 microliters. If a higher volume is injected, the animal can die immediately due to increased intracranial pressure. After application of the cells, the syringe was not removed immediately in order to allow dissolution of the cells in the brain.
15 After approximately 30 seconds, the syringe was removed slowly. Under these conditions no or very little liquid comes out of the burrhole. The skull was then cleaned and the animals did not bleed at the area operated (check via microscope). The skin was
20 then closed with sutures. Approximately 20-30 minutes was needed for intracerebral grafting of tumor cells in one animal (plus anesthesia).

Animals are monitored for 18-22 days at which time surviving rats are sacrificed, their brains
25 removed (quick frozen or fixed in formalin) and analyzed by standard techniques for measuring tumor volume.

Experiment No. 1

Number of Rats	Number of C6 Cells	Virus-Producer Cell Line	Number of Virus-Cells
8	5×10^4	Flk-1 TM	5×10^6
8	5×10^6	O	O

30

35

All cells were placed intracerebrally in a total
volume of ≤25 µl.

The results of this experiment are described in
Section 6.2.6 and shown in Figures 16A and 16B.

5

6.1.14. ASSAY FOR AND IDENTIFICATION OF ORGANIC COMPOUNDS THAT MODULATE FLK-1 MEDIATED SIGNAL TRANSDUCTION

Organic compounds that modulate Flk-1 receptor
10 mediated signal transduction can be assayed in a
cellular Flk-1 assay wherein modulation of Flk-1
receptor autophosphorylation is measured using an
antiphosphotyrosine antibody. In the example shown
below, the results are analyzed using a Western blot
15 of electrophoresed cell lysates. Levels of
phosphorylation as be measured by other techniques
known in the art.

NIH3T3 cells expressing high levels of Flk-1 were
seeded in 12-well plates at 250,000 cells/well in DMEM
20 + 0.5% calf serum and incubated overnight at 37° C
plus or minus the test substance. Flk-1 tyrosine
kinase was stimulated by the addition of 100-500 pM of
VEGF/well for 5-10 minutes at 37° C. After
stimulation, cells were washed with phosphate buffered
25 saline (PBS) and then lysed with 200 µl of sample
buffer (100 mM Tris pH 6.8, 5% glycerol, 1.75% SDS,
1.25 mM EDTA, 0.5 mM sodium vanadate, 2.5 mM sodium
pyrophosphate, 1.25% 2-mercaptoethanol). Cell lysates
were transferred to centrifuge tubes, boiled at 100° C
30 for 5 minutes, and then centrifuged at 16,000 G for 5
minutes. Supernatants were removed and stored at
-80° C.

For the Western blot assay, forty microliters of
saved supernatant per lane were loaded onto a 7.5%
35 SDS-PAGE gel (10 lanes/gel, 1.5 mm thick) and run at
120 V until the dye reached the bottom of the gel.

The running buffer used contains 20 mM Tris, 192 mM glycine and 0.1% SDS. Proteins were then transferred to nitrocellulose membrane (Bio-Rad) at 500 mA for 1 hour using ice-cold transblotting buffer containing

5   50% tank buffer, 20% methanol and 30% water. The nitrocellulose was then blocked with 5% nonfat milk in TBST (50 mM Tris, 150 mM NaCl, and 0.1% triton) for 1 hour or overnight, immunoblotted with a monoclonal antibody against phospho-tyrosine (UBI or Sigma,

10   1:3000) in TBST for 1 hour, followed by goat anti-mouse (Bio-Rad 1:3000) in TBST-buffer for another 1 hour. Protein bands were detected by soaking the membrane in ECL chemiluminescence system (Amersham Corp., prepared by mixing equal volumes of reagent 1

15   and 2) for 1 minute and then exposing the film for about 1-10 minutes.

6.1.15. SYNTHESIS OF A 3-PHENYL-1, 4-DIAZA-ANTHRACENE

20   A preferred method of synthesis of AG1385 is as follows: 0.47 grams (3mM) 2,3-diaminonaphthalene and 0.47 grams phenyl gloxal hydrate in 20 ml ethanol were refluxed 1.5 hour. Cooling and filtering gave 0.5g (65%) of a light brown solid, mp 163 °C. NMR CDC13: δ

25   9.38 (1H, l.c., H2), 8.71, 8.67 (2H, 2d, H5,10), 8.25, 8.10 (4H, AA'BB'm, H6-9), 7.58(5H, m, Ph). MS: +256(M+, 100%), 229 (M-CN, 12%), 126(71%) m/e.

6.2. RESULTS

6.2.1. ISOLATION OF Flk-1

30   To identify RTKs that are expressed during mouse development, PCR assays using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase

35   domain of RTKs were performed (Hanks, S.K. et al. 1988, Science 241:42-52). DNA extracted from a λgt10

cDNA library of day 8.5 mouse embryos (Fahrner, K. et
al., 1987, EMBO. J., 6:1497-1508), a stage in mouse
development at which many differentiation processes
begin was used as the template in the PCR assays. In
5   a parallel approach, with the intention of identifying
RTKs that regulate angiogenesis, similar primers were
used for the amplification of RTK cDNA sequences from
capillary endothelial cells that had been isolated
from the brains of postnatal day 4-8 mice, a time at
10   which brain endothelial cell proliferation is maximal
(Robertson, P.L. et al., 1985, Devel. Brain Res.
23:219-223). Both approaches yielded cDNA sequences
(FIG. 11, SEQ. ID NO.:) encoding the recently
described fetal liver RTK, Flk-1 (Matthews, W. et al.,
15   1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030).
Based on amino acid homology, this receptor is a
member of the type III subclass of RTKs (Ullrich, A.
and Schlessinger, J. 1990, Cell 61:203-212) and is
closely related to human *flt,* which also contains
20   seven immunoglobin-like repeats in its extracellular
domain in contrast to other RTKs of that subfamily,
which contain only five such repeat structures
(Matthews, W. et al., 1991, Proc. Natl. Acad Sci.
U.S.A. 88:9026-9030). Sequence comparisons of Flk-1
25   with KDR (Terman, B.I. et al., 1991, Oncogene 6:1677-
1683) and TKr-C (Sarzani, R. et al., 1992, Biochem.
Biophys. Res. Comm. 186:706-714) suggest that these
are the human and rat homologues of Flk-1,
respectively (Figure 1).
30

6.2.2   EXPRESSION OF Flk-1 mRNA
DURING EMBRYONIC DEVELOPMENT

As a first step towards the elucidation of the
biological function of Flk-1, the expression of Flk-1
35   mRNA was analyzed in mouse embryos at different

development stages. Northern blot hybridization
experiments indicated abundant expression of a major
5.5 kb mRNA between day 9.5 and day 18.5, with an
apparent decline towards the end of gestation (Figure

5   2A). In postnatal day 4-8 brain capillaries Flk-1
mRNA was found to be highly enriched compared to total
brain mRNA (Figure 2B).

    In situ hybridization experiments were performed
to obtain more detailed information about the

10   expression of Flk-1 during different embryonal stages.
A single-stranded antisense, 2619-nucleotide-long DNA
probe comprising the Flk-1 extracellular domain was
used as a probe because it generated the most specific
hybridization signals. As an example, a parasagittal

15   section of a day 14.5 embryo is shown in Figures 3A,
3b and 3C. High levels of hybridization were detected
in the ventricle of the heart, the lung, and the
meninges; other tissues such as brain, liver, and
mandible appeared to contain fewer cells expressing

20   Flk-1 mRNA. Thin strands of Flk-1 expression were
also observed in the intersegmental regions of the
vertebrae and at the inner surface of the atrium and
the aorta. Higher magnification revealed that the
expression of Flk-1 seemed to be restricted to

25   capillaries and blood vessels. Closer examination of
the heart, for example, showed positive signals only
in the ventricular capillaries and endothelial lining
of the atrium (Figure 4A). In the lung, Flk-1
expression was detected in peribronchial capillaries,

30   but was absent from bronchial epithelium (Figure 4D).
The aorta showed strong hybridization in endothelial
cells, but not in the muscular layer (Figure 4C).

35

6.2.3. EXPRESSION OF Flk-1
DURING ORGAN ANGIOGENESIS

The neuroectoderm in the telencephalon of a day
11.5 mouse embryo is largely avascular; the first
vascular sprouts begin to radially invade the organ
originating from the perineural vascular plexus (Bär,
J., 1980, Adv. Anat. Embryol. Cell. Biol. 59:1-62;
Risau, W. and Lemmon, V. 1988, Dev. Biol. 125:441-
450). At this stage, expression of Flk-1 was high in
the perineural vascular plexus and in invading
vascular sprouts, as shown in Figure 5A. These in
situ hybridization analyses indicated that the
proliferating endothelial cells of an angiogenic
sprout expressed the Flk-1 mRNA. At day 14.5, when
the neuroectoderm is already highly vascularized,
numerous radial vessels as well as branching vessels
of the intraneural plexus contained large amounts of
Flk-1 mRNA (Figure 5B). At postnatal day 4, when
sprouting and endothelial cell proliferation is at its
highest, strong expression of Flk-1 mRNA was observed
in endothelial cells (Figure 5C). Conversely, in the
adult brain when angiogenesis has ceased, Flk-1
expression was very low (Figure 5D) and appeared to be
restricted mainly to the choroid plexus (Figures 6A
and 6B). In the choroid plexus, cells in the inner
vascular layer expressed Flk-1 mRNA, while epithelial
cells did not (Figure 6A, 6B).

The embryonic kidney is vascularized by an
angiogenic process (Ekblom, P. et al., 1982, Cell
Diff. 11:35-39). Glomerular and peritubular
capillaries develop synchronously with epithelial
morphogenesis. In the postnatal day 4 kidney, in
addition to other capillaries, prominent expression of
Flk-1 was observed in the presumptive glomerular
capillaries (Figure 7A). This expression persisted in
the adult kidney (Figure 7C and 7D) and then seemed to

be more confined to the glomerular compared to the early postnatal kidney.

6.2.4. Flk-1 EXPRESSION IN ENDOTHELIAL CELL PROGENITORS

To investigate the possible involvement of Flk-1 in the early stages of vascular development, analysis of embryos at different stages during blood island formation were performed. In a sagittal section of the deciduum of a day 8.5 mouse embryo, Flk-1 expression was detected on maternal blood vessels in the deciduum, in the yolk sac and in the trophectoderm. Flk-1 mRNA was also found in the allantois and inside the embryo, mainly located in that part where mesenchyma is found (Figure 8A). At a higher magnification of the maternal deciduum, high levels of Flk-1 mRNA expression were found in the inner lining of blood vessels, which consist of endothelial cells (Figure 8B). In the yolk sac, hybridization signals were confined to the mesodermal layer, in which the hemangioblasts differentiate (Figure 8C). Figure 8D shows a blood island at higher magnification, in which the peripheral angioblasts expressed a high level of Flk-1 mRNA.

6.2.5. Flk-1 IS A HIGH AFFINITY RECEPTOR FOR VEGF

Detailed examination of in situ hybridization results and comparison with those for VEGF recently reported by Breier, G. et al. (1992, Development 114:521-532) revealed a remarkable similarity in expression pattern. Furthermore, Flk-1 expression in the glomerular endothelium and VEGF in the surrounding epithelial cells (Breier, G. et al., 1992, Development 114:521-532) raised the possibility of a paracrine relationship between these cells types and suggested

therefore a ligand-receptor relationship for VEGF and
Flk-1, respectively. In order to test this
hypothesis, the full-length Flk-1 cDNA was cloned into
the mammalian expression vector pCMV, which contains
5   transcriptional control elements of the human
cytomegalovirus (Gorman, C.M. et al., 1989, Virology
171:377-385). For transient expression of the
receptor, the Flk-1 expressing plasmid was then trans-
fected into COS-1 fibroblasts.
10   Specific binding of VEGF to the Flk-1 RTK was
demonstrated by crosslinking and competition binding
experiments. Purified ^{125}I-labeled VEGF was incubated
with COS-1 cells transfected with the pCMV-Flk-1
expression vector. Crosslinking with DSS and
15   subsequent analysis of immunoprecipitation, PAGE, and
autoradiography revealed an approximately 220 kD band
which was not detected in the control experiment with
untransfected COS-1 cells and is likely to represent
the VEGF/Flk-1 receptor complex (Figure 9A). In
20   addition, VEGF competed with ^{125}I-VEGF binding to Flk-1
expressing COS-1 cells (Figure 9B), whereas untrans-
fected COS-1 cells did not bind ^{125}I-VEGF. The
interaction of VEGF with the receptor on transfected
cells was specific, as PDGF-BB did not compete with
25   binding of ^{125}I-VEGF. Analysis of the binding data
revealed a Kd of about 10^{-10} M, suggesting that Flk-1
is a high affinity receptor of VEGF. This finding,
together with the Flk-1 and VEGF in situ hybridization
results strongly suggests that Flk-1 is a physiolo-
30   gically relevantly receptor for VEGF.
An autophosphorylation assay was performed to
confirm the biological relevance of VEGF binding to
the Flk-1 receptor. COS1 cells which transiently
expressed Flk-1 were starved in DMEM containing 0.5%
35   fetal calf serum for 24h, stimulated with 0.5 mM VEGF,

and lysed. The receptors were immunoprecipitated with
the Flk-1 specific polyclonal antibody CT128, and then
analyzed by SDS-PAGE and subsequent immunoblotting
using the antiphosphotyrosine antibody 5E2 (Fendly,
5   B.M. et al., 1990, Cancer Research 50:1550-1558). A
shown in Figure 10, VEGF stimulation of Flk-1
expressing cells led to a significant induction of
tyrosine phosphorylation of the 180 kD Flk-1 receptor.

10   6.2.6.   INHIBITION OF TUMOR GROWTH
                         BY TRANSDOMINANT-NEGATIVE
                         INHIBITION OF Flk-1

         The Flk-1 receptor is believed to play a major
role in vasculogenesis and angiogenesis. Therefore,
15 inhibition of Flk-1 activity may inhibit
vasculogenesis of a developing tumor, for example, and
inhibit its growth.
         The dominant-negative potential of Flk-1 TM was
first examined by measuring its influence on the
20 mitogenic response of Flk-1-expressing NIH 3T3 cells
after superinfection with the Flk-1 TM virus. [^{3}H]
thymidine incorporation in the 3T3 Flk-1 cell line was
maximally stimulated by 500 pM VEGF, with an EC_{50} of
about 100 pM. After superinfection with the Flk-1 TM
25 virus, the Flk-1/VEGF-mediated mitogenic response was
dramatically suppressed even at a ligand concentration
of 5 nM. While 3T3 Flk-1/Flk-1 TM cells expressed
wild type Flk-1 levels equal to the parental line,
they displayed, due to overexpression of Flk-1 TM, a
30 6-fold increase of cell surface receptors, as
determined by ^{125}I-VEGF binding. These results were
further extended by Flk-1 TM virus-induced suppression
of Flk-1 transforming activity and demonstrated not
only that mutant and wild type Flk-1 physically
35 associated, but also that this interaction generated
signalling-incompetent heterodimers. The dominant-

negative inhibitory effect which was achieved at a
fivefold excess of Flk-1 TM could not be overcome by a
50 fold ligand excess relative to the EC_{50} value for
mitogenic activation. Moreover, Flk-1 TM did not

5   interfere with the signal transduction of the α- and
β-PDGF-receptors, demonstrating the specificity of its
dominant-negative action.

To test the dominant-negative potential *in vivo*,
tumor cells (C6 rat glioblastoma) and mouse cells

10 producing a recombinant retrovirus encoding a
truncated Flk-1 receptor were mixed and implanted
subcutaneously into nude mice. The implanted C6
glioblastoma cells secrete VEGF which will bind to and
activate the Flk-1 receptors expressed on the surface

15 of mouse endothelial cells. In the absence of any
inhibitors of vasculogenesis, the endothelial cells
will proliferate and migrate towards the tumor cells.
Alternatively, if at the time of injection, the tumor
cells are co-injected with cells producing recombinant

20 retrovirus encoding the dominant-negative Flk-1, or if
the cells producing recombinant retrovirus are
injected after the tumor cells, the endothelial cells
growing towards the implanted tumor cells will become
infected with recombinant retrovirus which may result

25 in dominant-negative Flk-1 mutant expression and
inhibition of endogenous Flk-1 signaling. Suppression
of endothelial cell proliferation and migration will
result in failure of the implanted tumor cells to
become vascularized which will lead to inhibition of

30 tumor growth. As shown in Figures 12, 13 and 15 tumor
growth is significantly inhibited in mice receiving
implantations of cells producing truncated Flk-1
indicating that expression of a truncated Flk-1
receptor can act in a dominant-negative manner to

35 inhibit the activity of endogenous wild-type Flk-1.

As a control, any direct influence of the retroviruses on the growth of the tumor cells can be excluded by growing C6 cells in conditioned media of the different retrovirus-producing cell lines, without

5 any effect on their growth behavior.

To test the role that Flk-1 receptor is believed to play in angiogenesis and vasculogenesis, and to identify potential inhibitors of the Flk-1 receptor, tumor cells (C6 rat glioblastoma) and mouse cells

10 producing a recombinant retrovirus encoding a truncated Flk-1 receptor were mixed and implanted intracranially in rats. The implanted C6 glioblastoma cells secrete VEGF which will bind to and activate the Flk-1 receptors expressed on the surface of rat

15 endothelial cells. Inhibition of Flk-1 receptor signal transduction is measured as inhibition of intracranially tumor growth as seen in rats co-injected with the Flk-1 TM.

Analogous experiments with a variety of other

20 tumor types support the data obtained with the C6 glioblastoma nude mouse subcutaneous model and strongly demonstrate that inhibition of solid tumor growth can be effected by preventing angiogenesis. Angiogenesis a process that is normally regulated by

25 VEGF, which when secreted by tumor cells attracts and stimulated in a paracrine fashion Flk-1-positive vascular endothelial cells.

6.2.7. IDENTIFICATION OF AN ORGANIC COMPOUND THAT INHIBITS FLK-1
30 RECEPTOR PHOSPHORYLATION

To identify organic compounds that inhibit Flk-1 receptor, organic compounds have been tested in the cellular assay described in Section 6.1.14 for their ability to inhibit Flk-1 receptor phosphorylation.

35

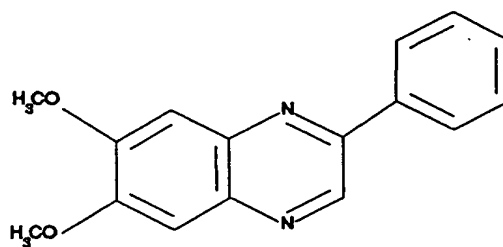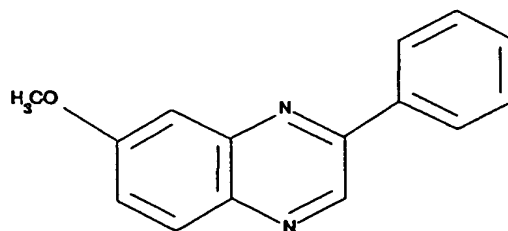Examples of some of the compounds tested are shown below.
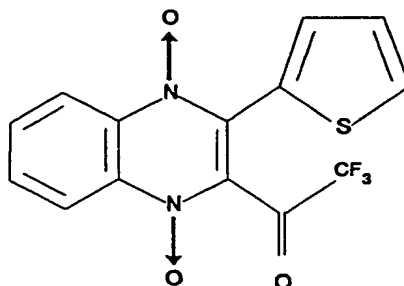
5
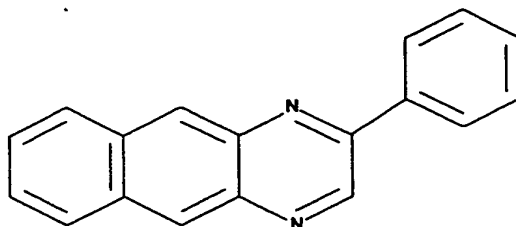
A14

15

A9

20

25

A2

30

35

5

A5

10

Compound A14 was shown to inhibit Flk-1 receptor
phosphorylation almost completely at a test
concentration of 100 μM (Figure 17). Thus this is an
example of a compound that could be useful for
15 antagonizing Flk-1 receptor signal transduction and
therefore may be useful for inhibiting the Flk-1
receptor mediated mitogenic signal. Accordingly,
compound A14 may be therapeutically useful in the
treatment of solid tumors by inhibiting angiogenesis.
20   The present invention is not to be limited in
scope by the exemplified embodiments which are
intended as illustrations of single aspects of the
invention, and any clones, DNA or amino acid sequences
which are functionally equivalent are within the scope
25 of the invention. Indeed, various modifications of
the invention in addition to those described herein
will become apparent to those skilled in the art from
the foregoing description and accompanying drawings.
Such modifications are intended to fall within the
30 scope of the appended claims.
     It is also to be understood that all base pair
sizes given for nucleotides are approximate and are
used for purposes of description.
     All references cited herein are hereby
35 incorporated by reference in their entirety.